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Review

Signaling pathways effecting crosstalk between cartilage and adjacent tissues
Seminars in cell and developmental biology: The biology and pathology of cartilage

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ABSTRACT

Endochondral ossification, the mechanism responsible for the development of the long bones, is dependent on an extremely stringent coordination between the processes of chondrocyte maturation in the growth plate, vascular expansion in the surrounding tissues, and osteoblast differentiation and osteogenesis in the perichondrium and the developing bone center. The synchronization of these processes occurring in adjacent tissues is regulated through vigorous crosstalk between chondrocytes, endothelial cells and osteoblast lineage cells. Our knowledge about the molecular constituents of these bidirectional communications is undoubtedly incomplete, but certainly some signaling pathways effective in cartilage have been recognized to play key roles in steering vascularization and osteogenesis in the perichondrial tissues. These include hypoxia-driven signaling pathways, governed by the hypoxia-inducible factors (HIFs) and vascular endothelial growth factor (VEGF), which are absolutely essential for the survival and functioning of chondrocytes in the avascular growth plate, at least in part by regulating the oxygenation of developing cartilage through the stimulation of angiogenesis in the surrounding tissues. A second coordinating signal emanating from cartilage and regulating developmental processes in the adjacent perichondrium is Indian Hedgehog (IHH). IHH, produced by pre-hypertrophic and early hypertrophic chondrocytes in the growth plate, induces the differentiation of adjacent perichondrial progenitor cells into osteoblasts, thereby harmonizing the site and time of bone formation with the developmental progression of chondrogenesis. Both signaling pathways represent vital mediators of the tightly organized conversion of avascular cartilage into vascularized and mineralized bone during endochondral ossification.

Contents

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1. Introduction

All the long bones of the mammalian skeleton originate from cartilage templates via a complex multistep process termed endochondral ossification. During this process, mineralized bone is formed through the deposition of bone matrix by differentiated osteoblasts on top of a scaffolding cartilage mold generated by chondrocytes. This mold or cartilaginous bone template is formed during early fetal limb development by cells within the mesenchymal condensations that commit to the chondrocyte lineage under the influence of SOX9, the master transcriptional regulator of chondrogenesis [1]. Adequate longitudinal growth of the endochondral bones is absolutely dependent on precisely regulated proliferation, differentiation and matrix production by chondrocytes in the cartilaginous condensations and later in the growth plate. In addition, proper progression of bone development relies on a very stringent coordination between the processes of chondrocyte maturation in the growth plate, vascular expansion in the surrounding tissues, and osteoblast differentiation, recruitment, and bone-forming activity in the perichondrium and the developing bone center. The synchronization of these processes occurring in these adjacent tissues is regulated through vigorous crosstalk between chondrocytes, endothelial cells and osteoblast lineage cells. Studies using a growing number of genetically modified mouse models are increasingly providing insights in the molecular interplay regulating these complex interactions, some of which will be reviewed here (Fig. 1).

2. Chondrocyte-driven regulation of angiogenesis in surrounding tissues

During endochondral ossification, chondrocytes first generate cartilaginous templates of the forming bones from mesenchymal condensations, and subsequently create the growth plates that provide the prime engine for bone growth. These cartilaginous structures are rather unique tissues in that they are inherently avascular and physiologically hypoxic [2–4]. Hypoxia-driven pathways, governed by transcription factors called hypoxia-inducible factors (HIFs), are absolutely essential for the survival and functioning of chondrocytes in these challenging conditions [4] (see Fig. 2). HIF-mediated signaling has also been implicated in joint formation and the integrity of the adult articular cartilage [5,6]. One of the mechanisms by which HIF supports cartilage development is through the regulation of angiogenesis in the adjacent perichondrial tissues, mediated by the potent angiogenic stimulator vascular endothelial growth factor (VEGF) that is a direct transcriptional target of HIF (see Fig. 2). VEGF is also a key driver of the progressive conversion of the prefiguring cartilage into bone tissue during skeletal development and growth, a process that is driven by cartilage neovascularization and the concomitant infiltration of the future ossified region by osteoprogenitors (see Fig. 3). Here, we will go deeper into the studies performed to dissect the roles of hypoxia pathway components and VEGF family members during these key stages of the endochondral ossification program.

2.1. The multiple roles of the hypoxia-HIF-VEGF network in avascular cartilage

2.1.1. Hypoxia-inducible factors (HIFs)

Cartilage, being an intrinsically avascular tissue, is highly dependent on cellular hypoxia-adaptation mechanisms. HIFs, the transcription factors that are the main orchestrators of the cellular responses to hypoxia, act as heterodimers consisting of an α-subunit which is regulated by oxygen (HIF-1α, HIF-2α/EPAS1, or the less characterized HIF-3α), and a β subunit that is constitutively expressed in an oxygen-independent manner. The best characterized member, HIF-1, is formed in hypoxic conditions by the subunits HIF-1α and HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT)), which both contain basic helix-loop-helix-PAS domains that mediate heterodimerization and DNA binding [7–9].

In well-oxygenated conditions, the HIF-1α protein is hydroxylated on specific residues within its amino-acid sequence (prolines P402 and P564 in the oxygen-dependent degradation domain (ODD)), in an oxygen-dependent reaction executed by HIF prolyl-hydroxylase enzymes (PHD1-3, also known as Egln2, 1, and 3) [10]. Hydroxylated HIF-1α is recognized by the Von Hippel-Lindau protein (pVHL), which is part of an E3 ubiquitin ligase complex, leading to the ubiquitination and instant proteosomal degradation of HIF-1α in non-hypoxic conditions [7–9].

In hypoxia, represented by oxygen tension levels dropping below an estimated threshold of 5%, the hydroxylation and degradation of HIF-1α is inhibited. As HIF-1α is stabilized in these conditions, the protein can translocate to the nucleus and dimerize with HIF-1β. Along with nuclear co-factors such as p300 and CREB-binding protein (CBP), HIF-1α and HIF-1β form the transcriptional complex HIF-1. The complex can bind to hypoxia responsive elements (HRE), present in the promoter region of target genes, and induce transcription. More than a hundred putative HIF-1 target genes have been identified, several of which function in cell proliferation, differentiation, survival, resistance to oxidative stress, apoptosis, and extracellular matrix homeostasis, but most typically and abundantly involving genes regulating energy metabolism and

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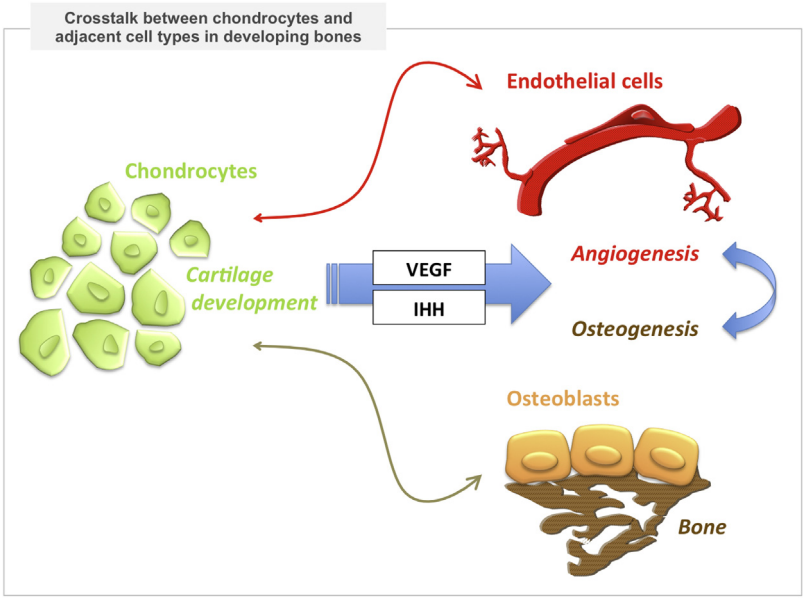

Fig. 1. Crosstalk between cartilage and adjacent tissues, focusing on chondrocyte-derived factors regulating angiogenesis and osteogenesis in the surrounding perichondrium and bone center. Abundant crosstalk and interplay between chondrocytes, endothelial cells, and osteoblasts ensures the proper synchronization of endochondral bone development and growth. VEGF and IHH represent two key cartilage-derived signaling molecules serving critical coordinating roles in endochondral ossification by inducing angiogenesis and osteogenesis in the tissues surrounding the avascular cartilage. Conversely, endothelial cells and osteoblast lineage cells release angiocrine and osteocrine factors, respectively, which in addition to regulating angiogenic-osteogenic coupling may also affect the differentiation or fate of adjacent chondrocytes. The molecular constituents that mediate this reciprocal communication are only beginning to be unraveled.

angiogenesis [7–9]. Indeed, the major overall goal of the hypoxia-response is increasing the oxygen delivery to hypoxic tissues while decreasing their oxygen consumption. HIFs hereby function as master regulators of oxygen homeostasis through their combined transcriptional actions on VEGF, stimulating angiogenesis, erythropoietin (EPO), activating erythropoiesis, and glycolytic enzymes involved in anaerobic metabolism. The latter mediate the cell's adaptation to hypoxia by reducing its oxygen consumption in glucose utilization pathways and its dependence on oxygen to generate ATP. The shift towards glycolysis at the same time prevents the generation of excess reactive oxygen species (ROS) in the mitochondria due to inefficient electron transport under hypoxic conditions [7–9].

2.1.2. *Hypoxia-driven, VEGF-mediated angiogenesis in cartilage-surrounding tissues*

A myriad of mouse genetics studies performed over the past 15 years support the essential roles of HIF-1 and VEGF in chondrocyte biology and the regulation of the development and growth of the long bones. Given the early fetal lethality of ubiquitous knockout mice for these important molecules, insights in skeletal biology have been gained predominantly by employing selective knock-in mice and Cre-loxP strategies to generate conditional knockout (cKO) models. Particularly the use of the Prx1-Cre- and type II collagen (Col2)-Cre driver strains, targeting respectively the fetal limb bud mesenchyme or endochondral osteochondroprogenitors and chondrocytes, has been instrumental in defining the functions of the hypoxia-regulated pathways and specific molecular components thereof in endochondral bone development.

The first study that revealed the essential physiological role of hypoxia-driven pathways in cartilage used a Col2-Cre driven conditional deletion model of HIF-1α, showing that loss of HIF-1α in chondrocytes led to massive cell death in the developing growth plate, resulting in dwarfism with marked shortening of the limbs [4]. Specifically, the chondrocytes located in the center of the growth cartilage, farthest from the perichondrial blood vessels, died in the absence of HIF-1α. These regions co-localized with the presence of hypoxia, as detected by a marker for bioreductive activity (EF5) [4]. Since this pioneering work, several studies have increasingly shed light on the mechanisms by which HIF-1α ensures the survival of hypoxic chondrocytes, including the direct activation of genes that enable chondrocytes to switch to oxygen-sparing metabolic pathways (see further) and the indirect consequences of induction of VEGF [2,11] (Fig. 2).

VEGF is one of the most powerful and critical mediators of angiogenesis. The deletion of even a single copy of the *VEGF* gene resulted in early embryonic lethality due to defective vascular development [12,13]. Interestingly, mutant mice in which VEGF was deleted in cartilage showed massive apoptosis of non-hypertrophic, hypoxic chondrocytes in the interior of the developing growth plate [2,14,15], similar to what was seen with HIF-1α deficiency [4]. Since the expression and protein stability of VEGF is regulated by hypoxia at multiple levels, including a direct transcriptional activation by HIF-1α [16–19], these findings suggested that VEGF is downstream of HIF-1α and that the hypoxia-HIF-VEGF network provides a vital pathway supporting chondrocyte survival in the avascular growth plate during bone development.

Several observations indicate that the prime function of cartilage-derived VEGF is to stimulate angiogenesis in the immediate surrounding perichondrial tissues, thereby increasing the oxygen supply into the growing cartilage and lessening the degree of hypoxia to prevent cell death (Fig. 2). Indeed, mice lacking VEGF in cartilage (Col2-Cre driven) displayed impaired vascularization surrounding the epiphysis and increased hypoxia within the cartilage [2]. These phenotypic manifestations, as well as the aberrant apoptosis of centrally located chondrocytes, could be rescued completely by transgenic over-expression of VEGF164 in the Col2-expressing cells, which induced excessive perichondrial angiogenesis [2]. VEGF164 is one of the major splice isoforms of VEGF, which are derived from a single *VEGF* gene through alternative splicing of the pre-mRNA. Five VEGF isoforms have been identified in humans, while there are three major isoforms in the mouse: VEGF120, VEGF164, and VEGF188. The shortest isoform, VEGF120, lacks the basic, heparin-binding residues encoded

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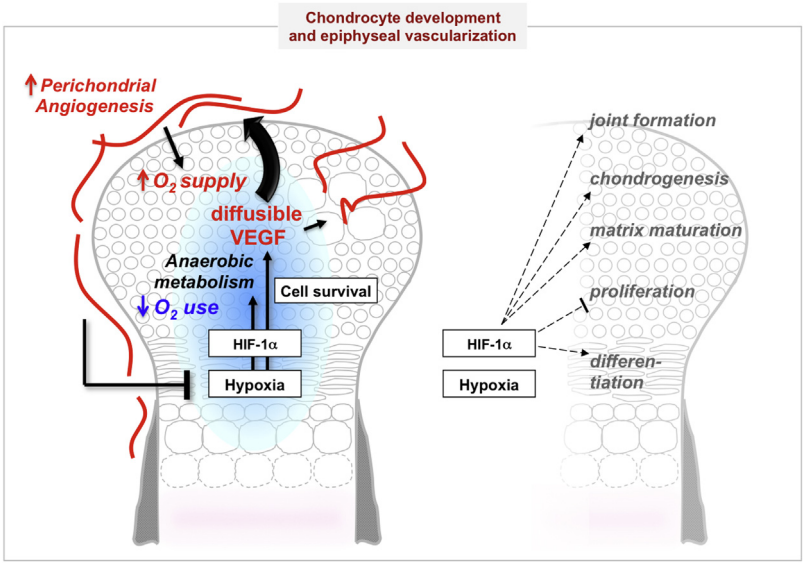

Fig. 2. The role of the HIF—VEGF network in developing cartilage. (Left) Hypoxia-driven, VEGF-mediated angiogenesis in cartilage-surrounding tissues. Avascular cartilage exists as a physiological hypoxic tissue during bone development and growth. A gradient of reducing oxygenation emanates from the vascularized peripheral tissues towards the interior of the growth plate, which inherently becomes hypoxic as the cartilage element grows (blue area). Hypoxia, at least in part via HIF-1α, induces expression of VEGF by the chondrocytes. The soluble VEGF isoforms VEGF120 and VEGF164, but not the matrix-bound isoform VEGF188, can diffuse to the perichondrial tissues around the cartilage and stimulate angiogenesis, increasing the O_2 supply into the cartilage and limiting the degree of hypoxia. HIF-1 additionally serves to switch the energy metabolism of the chondrocytes towards anaerobic glycolysis, consequentially reducing the use of oxygen. By controlling oxygen availability and consumption, the hypoxia-HIF-VEGF network ensures the survival of the hypoxic chondrocytes in the avascular growth cartilage. At a later stage, hypertrophic chondrocytes appear within the epiphyseal cartilage and become the site of invasion by vascular canals; diffusible VEGF signaling is involved in attracting blood vessels into the epiphysis and launching secondary ossification center formation. (Right) Additional roles of hypoxia-driven pathways in cartilage development. In the avascular cartilage, hypoxia and HIF-1α affect chondrocyte development and cartilage matrix maturation at multiple steps, as indicated in the scheme. For details, see text. Figure adapted from [21,177].

by exons 6 and 7, whereas VEGF164 lacks the residues encoded by exon 6. This confers upon the secreted proteins differences in matrix binding, and is thought to create a gradient of VEGF for directional recruitment or growth of endothelial cells during angiogenesis: VEGF188 is mostly sequestered in the matrix, from where it can be released by proteases during matrix degradation, while VEGF120 fails to bind heparin and can diffuse to a more distant site. VEGF164 has intermediate matrix-association/solubility properties, and can furthermore bind the co-receptors neuropilin (NRP)-1 and NRP-2, in addition to the main receptor tyrosine kinases VEGFR-1 and VEGFR-2 [20,21]. These characteristics render this isoform particularly effectual, as knock-in mice in which the endogenous 8-exon-comprising VEGF gene was replaced by the cDNA encoding only the VEGF164 isoform showed no noticeable phenotypic alterations [22–24], including normal development of the endochondral bones [14]. In contrast, expression of exclusively the matrix-binding VEGF188 isoform recapitulated the impaired vascularization surrounding the epiphysis, aberrant hypoxia within the cartilage, and central chondrocyte apoptosis phenotype of mice conditionally lacking VEGF (all isoforms) or HIF-1α in cartilage [14], whereas expression of only the freely soluble VEGF120 isoform did not [25]. These findings indicate that soluble VEGF isoforms are critically required to stimulate vascularization in the perichondrial tissues surrounding the avascular cartilage, presumably due to their ability to diffuse to the surface of the cartilage. Thus, through the expression and secretion of hypoxia-induced VEGF, chondrocytes instruct the expansion of the epiphyseal vascular network in coordination with the growth of the avascular cartilage mass, in order to ensure sufficient oxygen supply to support chondrocyte survival (Fig. 2).

Interestingly, also in the early skeletal patterning stages during embryogenesis, VEGF secreted by the limb bud mesenchyme of the forming cartilaginous condensations appeared to act via a long-ranging mechanism (without involvement of receptor signaling in the condensations proper) to mediate the rearrangements in the vascular plexus in the surrounding tissues [26,27].

Notably, over-expression of transgenic VEGF164 in cartilage lacking HIF-1α could rescue vascularization in the surrounding tissues, but failed to completely restore the oxygen levels in the cartilage and only partially prevented the cell death phenotype [2]. The reason for this was found in a second vital mechanism downstream of HIF-1α, independent of VEGF, in the regulation of the metabolic adaptation of the chondrocytes when they become hypoxic during growth of the cartilaginous tissue mass. To support their survival, HIF-1α is absolutely essential to reprogram energy metabolism in the chondrocytes towards the use of oxygen-sparing anaerobic pathways [2]. This is achieved through induction of the expression of classical HIF target genes that mediate glycolysis, such as the enzyme phosphoglycerate-kinase 1 (PGK1), or that impair mitochondrial respiration, such as pyruvate dehydrogenase kinase 1 (PDK1), which shunts pyruvate away from the mitochondrial tricarboxylic acid cycle [2,4,28]. These adaptations serve to limit the oxygen consumption within cartilage.

Thus, HIF-1α regulates a fine balance of oxygen delivery and utilization in this challenged avascular tissue, to maintain the level of hypoxia within the delicate optimal range for chondrocytes to survive and develop and function normally [2] (Fig. 2).

2.1.3. Other roles of HIFs in chondrogenesis and cartilage development

Besides its prime function in mediating chondrocyte survival in their hypoxic environment, HIF-1α also regulates multiple other aspects of chondrocyte biology in mesenchymal condensations and developing growth plates (Fig. 2, right panel), as indicated by the study of a number of mutant mouse models. For instance, loss of HIF-1α in the early limb bud mesenchyme (mediated via the use of Prx1-Cre mice) did not seem to affect the initial formation of the mesenchymal condensations, but delayed the specification of the joints, the differentiation of mesenchymal cells into chondrocytes,

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and their terminal transition to hypertrophic chondrocytes [3,29]. A myriad of reports have substantiated that low oxygen tension promotes differentiation of mesenchymal stem cells into chondrocytes in vitro [30–36]. Interestingly, HIF-1α can induce expression of the master chondrogenic transcription factor SOX9, suggesting that the function of HIF-1α as an early differentiation factor in chondrogenesis may be mediated through SOX9 [29,37].

Some of the generated conditional knockout mice also indicated that HIF-1α negatively regulates the proliferation of chondrocytes in the developing growth cartilage (Fig. 2). In cartilage lacking HIF-1α (Col2-Cre driven), the proliferation rate of the viable chondrocytes in the peripheral regions of the fetal growth plate was strikingly increased [4]. Conversely, the chondrocyte proliferation rate was markedly reduced in mice with conditional inactivation of VHL (the E3 ubiquitin ligase that targets HIF-1α and HIF-2α to the proteasome for degradation and thus serves as a negative regulator of HIF activity) in cartilage or limb buds, which was associated with an increase of the cyclin-dependent kinase inhibitor p57 mRNA. The loss of VHL in chondrocytes in vivo thereby resulted in a pronounced hypocellularity in the growth plates and the mutant mice displayed severe dwarfism [38,39]. The inhibiting effect of HIF-1α on chondrocyte proliferation could further contribute to its actions to lower the oxygen consumption in the hypoxic tissue.

Another emerging function of HIF signaling in cartilage is in the control of the cartilaginous matrix accumulation and maturation (Fig. 2). Loss of VHL in cartilage or in the mesenchymal cells of the limb buds appeared to be associated with an increased accumulation of matrix in between the chondrocytes [38,39]. The relationship between hypoxia sensing and extracellular matrix (ECM) accumulation in cartilage involves a role for HIF-1α in improving the efficiency of post-translational modifications of type II collagen. These post-translational modifications, required for the acquisition of the mature triple helix collagen structure, include critical hydroxylation reactions of proline residues in the collagen proteins, which are catalyzed by a family of collagen prolyl-4-hydroxylases (cP4Hs, in particular cP4HaI and cP4HaII). Of note, these enzymes are distinct from the family of prolyl-hydroxylases that hydroxylate HIFs (the PHDs, introduced above) but also function in an oxygen-dependent manner by using oxygen as a substrate for proline hydroxylation [40]. The expression of cP4Hs in chondrocytes is induced through HIF-1α-dependent transcriptional stimulation [19,28,41]. This molecular link may explain the role of HIF-1α in facilitating the correct folding of the collagen proteins in the endoplasmic reticulum (ER) and preventing ER stress, and in increasing the secretion and accumulation of mature type II collagen in the cartilage ECM [16,28,38]. Moreover, accumulation of inappropriately processed collagen in the absence of HIF-1α could trigger the unfolded protein response (UPR), lead to ER stress and cause cell death, suggesting that hypoxia-induced cP4H-activity may contribute to the chondrocyte survival function of HIF-1α [28]. Recent evidence seems to support this idea, as growth plates from mice with an almost 70% decrease of cP4Hs enzymatic activity displayed an inner cell death phenotype reminiscent of that observed in growth plates lacking HIF-1α, albeit much milder and transient [42]. The mechanisms underlying the severe matrix abnormalities and chondrodysplasia observed in these mice ($cP4HaI^{fl/-}$;$cP4HaII^{-/-}$ double mutant mice, combining heterozygous inactivation of cP4HaI with homozygous inactivation of cP4HaII) will further help elucidating these important actions in the growth plate cartilage [5,11,42].

2.1.4. HIF-2α and the role of hypoxia in articular cartilage and joint pathophysiology

Although HIF-1α and HIF-2α both dimerize with HIF-1β and share extensive structural homology (48% homology in overall amino acid sequence, including some highly conserved important domains in the proteins), they do not exhibit the same distribution, target genes and functions, and they are consequently non-redundant. Distinct from HIF-1α, HIF-2α is not critical for joint specification during embryogenesis development or for growth plate development. Indeed, lack of HIF-2α was found to cause only a modest and transient delay of endochondral bone development in several mouse models. Heterozygous mouse embryos (ubiquitously lacking one allele of HIF-2α, i.e. $HIF\text{-}2\alpha^{+/-}$ fetuses) develop a mild and transient dwarfism that resolves by the age of two weeks [43]. Similarly, conditional inactivation of HIF-2α in the limb bud mesenchyme results in a mild and transient delay in endochondral bone development at E17.5 [44]. The molecular mechanisms responsible for this phenotype are still largely unknown, but may involve an impairment of the latest stage of chondrocyte hypertrophy in the fetal growth plate. HI plate. HIF-2α was identified as an inducer of the expression of typical proteins made by hypertrophic chondrocytes that play key roles in endochondral ossification, such as type X collagen, VEGF, and the matrix-degrading enzyme matrix metalloproteinase (MMP)-13 [43].

In contrast to its limited importance in cartilage during development, HIF-2α does appear to play a significant role in the articular cartilage of the postnatal joint and in the pathogenesis of osteoarthritis (OA). Articular cartilage represents an avascular, aneural, and alymphatic tissue, which relies for its nutrient supply and oxygenation on diffusion from the synovial fluid and the subchondral bone. The limited diffusion capacity of oxygen has been reported to create a gradient of 6% oxygen at the joint surface to only 1% in the deeper layers (reviewed in [45]). Articular chondrocytes consequentially face an hypoxic environment throughout life, and are very well adapted to these challenging conditions by the relatively constitutive expression of HIFs [45]. Both HIF-1α and HIF-2α are expressed in human articular surface chondrocytes [29,35,37,46], suggesting that their actions may contribute to the maintenance of the stable articular chondrocyte phenotype. However, increased expression of both factors has been found in human OA cartilage. The mRNA expression of HIF-1α and its target genes was elevated and correlated positively with OA progression and the severity of cartilage degradation [47]. HIF-2α levels were also higher in surgical specimens of OA patients and osteoarthritic mice compared with non-diseased cartilage [43,48]. The functioning of the respective HIF α subunits in OA cartilage, however, appears to differ significantly, at least in animal models. While HIF-1α has been postulated to be protective against OA and possibly drive compensatory mechanisms to conserve articular cartilage integrity during OA development, HIF-2α appears to be a catabolic regulator, mediating osteoarthritic cartilage matrix breakdown in OA [6]. In line therewith, inhibition of HIF-1α in the knee joint of normal healthy mice (by intra-articular injection of 2-methoxyestradiol) led to chondrocyte apoptosis and osteoarthritic changes, including articular cartilage degeneration and osteophyte formation [49]. In contrast, HIF-2α haplo-insufficiency in *Epas1*$^{+/-}$ mice prevented cartilage degradation and osteophyte formation after surgically induced instability of the knee joint, thus protecting the mice against OA [43,48]. Another loss-of-function study developed cartilage-targeting nanoparticles to deliver small-interfering RNA (siRNA) silencing HIF-2α expression to chondrocytes, which was associated with a decrease in HIF-2α, catabolic factors and VEGF in vitro. Upon intra-articular injection of the nanoparticles into the knee joints of OA-affected mice, cartilage integrity was maintained and synovial inflammation alleviated [50]. Conversely, gain-of-function of HIF-2α, obtained genetically or via adenovirus-mediated overexpression of the *Epas1* gene following local injection in the mouse knees, induced expression of the catabolic HIF-2α target genes and triggered severe cartilage destruction [48]. The upstream mechanisms leading to aberrant HIF-2α activity in OA appear to involve nuclear factor (NF)-κB signaling, itself activated

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by mechanical stress and/or proinflammatory cytokines, rather than HIF-2α regulation through PHD activity [43,48,51]. Thus, several animal models implied a pathophysiological role for HIF-2α in OA. A role for HIF-2α in human OA was suggested as well, as a functional single nucleotide polymorphism (SNP) in the human *EPAS1* gene encoding HIF-2α was found to associate with knee OA in a Japanese cohort [43], although this genetic link could not be recapitulated in all patient cohorts [52].

The role of hypoxia and HIFs, acting in conjunction with inflammatory pathways, in rheumatoid arthritis is complex and beyond the scope of this review; for more information on this subject, see [53].

2.2. The central role of angiogenic signaling by VEGF in the conversion of cartilage to bone

In the previous section, mechanisms were discussed by which the immature cartilage of the cartilaginous condensations and the developing growth plate sustain as avascular tissues, including distant actions of VEGF to mediate the growth of the vascular network in the surrounding tissues, without invading the cartilage proper. Different from immature chondrocytes, however, terminally differentiated hypertrophic chondrocytes allow and even trigger blood vessels to invade their matrix, a process that is inherently associated with decay of the hypertrophic cartilage and its replacement by bone. Throughout the endochondral ossification process, three key phases can be discriminated where vascular invasion of hypertrophic cartilage initiates the conversion of avascular cartilage into highly vascularized bone and bone marrow tissue, cumulating in the formation of the mature long bone. First, the initial invasion of the cartilage anlagen during embryonic development involves endothelial cells and osteoprogenitors co-invading from the perichondrial tissues and organizing the primary center of ossification. Second, continual capillary invasion at the metaphyseal border of the growth cartilage mediates progressive bone lengthening. Third, vascularization of the epiphyseal cartilage at the ends of the bone initiates the formation of secondary ossification centers. The central role of hypertrophic cartilage-derived VEGF as inducer of these consecutive vascularization and ossification events in the expanding bone centers will be briefly reviewed here; for more extensive reviews see [21,54].

2.2.1. VEGF controls the initial osteo-angiogenic invasion of the endochondral bone template

Around embryonic day 14 (E14) in the mouse, the chondrocytes located in the center (diaphysis) of the cartilage anlagen become hypertrophic and calcify their extracellular matrix. At this time, cells in the surrounding perichondrium differentiate into osteoblasts, at least in part under the influence of IHH signaling from the cartilage (see below), and form a mineralized bone collar, which constitutes the provisional cortical bone shaft. Besides osteogenic cells, also blood vessels and blood-borne osteoclasts increasingly accumulate in the perichondrial region. Subsequently, they invade the mid-diaphyseal hypertrophic cartilage core, degrade the cartilage matrix, and erode the region to make room for osteoblasts and bone marrow cells to populate the newly forming bone center. The hematopoietic precursors migrate from the fetal liver to establish marrow-based hematopoiesis.

As such, the initial vascular invasion of cartilage launches the formation of the primary ossification center or primitive bone marrow cavity of the long bone. It was shown, by using lineage-tracing approaches in mice, that the blood vessel invasion is closely associated with the migration of perichondrial osterix (Osx)-expressing osteoprogenitors into the nascent bone center, with a subset of the osteoprogenitors co-invading the tissue while positioning as pericytes around the blood vessels [55]. This coupled osteo-angiogenic invasion process may provide a partial explanation for the fact that cartilage neovascularization is an absolute requirement for the endochondral replacement of cartilage by bone, as supported by experiments physically blocking the angiogenic invasion process [56].

Several mouse models support the notion that VEGF plays critical roles in the recruitment of perichondrial blood vessels and the initial angiogenic invasion of the cartilage anlagen, and that these represent absolutely critical steps in the early development of the long bones [2,14,25,56–59]. At this time in development, endothelial cells, osteoblasts, and osteoclasts – the cell types that accumulate in the perichondrium surrounding the hypertrophic cartilage and all express VEGF receptors (VEGFRs) (see below) – coordinately invade the cartilaginous template [21,55] (Fig. 3A). Hypertrophic chondrocytes produce high levels of VEGF, a feature that is characteristically embedded in the terminal chondrocyte differentiation program and that is incompletely understood at the molecular level at present, although several factors have been implicated and may cooperatively regulate the VEGF induction. These include the transcription factors runt-related transcription factor 2 (Runx2) and Osx [60–62], which are primarily known for their essential roles in osteoblast differentiation (see below) but are also expressed in hypertrophic and pre-hypertrophic chondrocytes, respectively. Hypoxia too is a potent inducer of VEGF expression in chondrocytes, as outlined in the previous section; yet, it is not fully clear to what degree hypoxia and HIF-1α contribute to the high levels of VEGF production by late hypertrophic chondrocytes in the pre-invasion bone template and in the growth plate. One study suggested that Runx2 might be involved in the protein stabilization of HIF-1α and induction of VEGF in hypertrophic chondrocytes [63]. Moreover, in vivo observations do underscore a potential hypoxia- and HIF-1α-mediated control mechanism of the VEGF expression in the pre-invasion cartilage anlagen (Fig. 3A) [2,3,29]. Indeed, mice lacking either HIF-1α or VEGF in cartilage showed a delay in the initial invasion and primary ossification center formation, which could be rescued by forced VEGF164 overexpression in both mutants [2]. Also at this specific, early stage in the bone development process, the VEGF164 isoform proved to be of particular importance, as embryos expressing only the VEGF120 isoform or only the VEGF188 isoform both showed a delay in the early vascular invasion and development of the long bones [14,25]. Conversely, in mice over-expressing VEGF164 in the endochondral skeleton, achieved by a Col2-Cre-mediated conditional over-expression model, the osteo-angiogenic invasion of developing bone centers occurred prematurely and excessively, with aberrant bone deposition in this vascularized area leading to misshapen limbs [64]. These findings strongly suggest that hypoxia-induced, HIF-mediated VEGF expression in cartilage is required for the recruitment of angiogenic blood vessels into developing endochondral bone centers (Fig. 3A).

The concomitant recruitment and invasion by osteoclasts and osteoprogenitors may be in part explained as well by VEGF's cell-autonomous effects on these cell types. VEGF is a homodimeric glycoprotein of 45 kDa that belongs to the dimeric cysteine-knot growth factor superfamily. It binds to and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), whose prime role is to regulate angiogenesis, both in physiological and pathological conditions [20]. However, besides endothelial cells that are the principal targets of VEGF, numerous other cell types express VEGF receptors and respond to VEGF signaling [65,66], including osteoprogenitors, osteoblasts and osteoclasts [21,58]. Overall, VEGF can promote or modulate chemotaxis, proliferation, differentiation, survival and/or activity of a range of non-endothelial cell types, including osteoblasts and osteoclasts. While in most circumstances VEGF functions as a paracrine mediator, autocrine and intracrine roles have been described as well,

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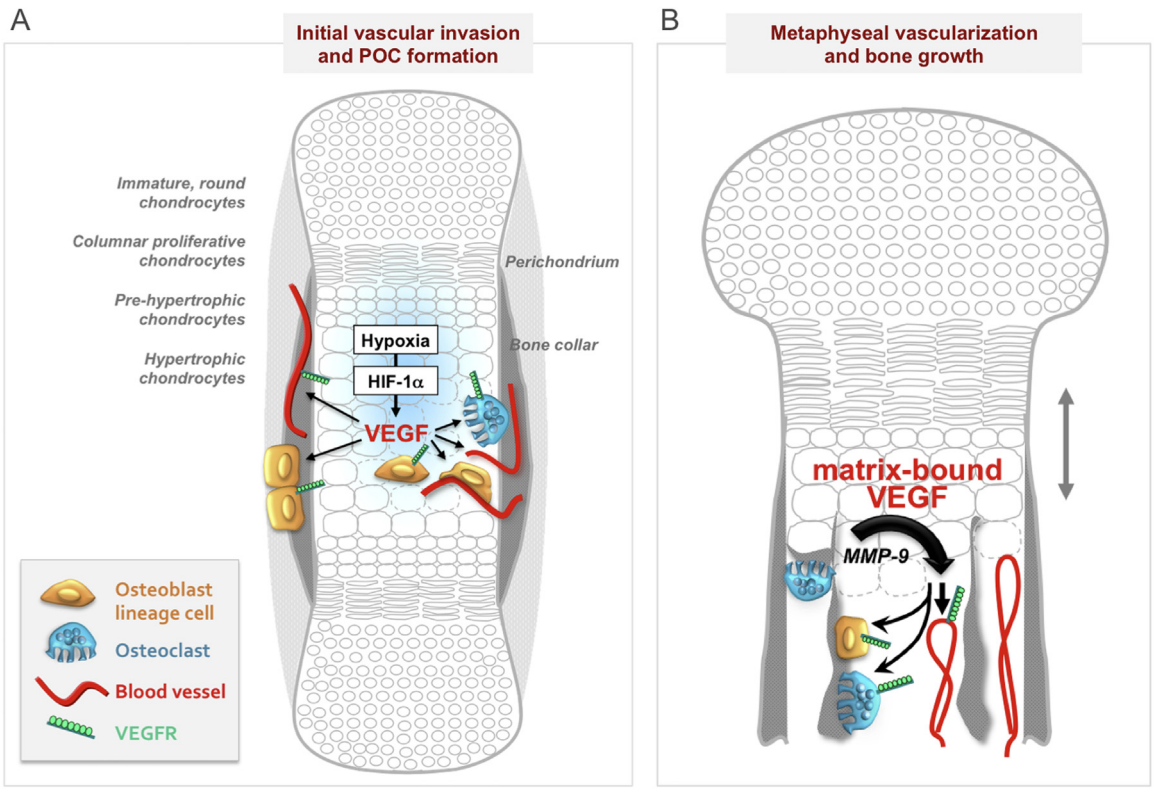

Fig. 3. The role of HIF and VEGF in the neovascularization of cartilage and its conversion to bone. (A) VEGF controls the initial osteo-angiogenic invasion of the endochondral bone template. During long bone development, hypertrophic chondrocytes in the middle diaphyseal region of the avascular cartilage template become hypoxic (blue shading) and express high levels of VEGF. Both HIF-1α and VEGF are required for the timely invasion of the template by blood vessels from the perichondrium. Along with the endothelium, osteoprogenitors move into the tissue, start to deposit bone, and establish the primary ossification center. Osteoclasts, cells of hematopoietic origin, also appear coinciding with vascular accumulation in the perichondrium and co-invade the cartilage. All the cell types involved express VEGF receptors (VEGFR) and can respond directly to VEGF signaling by enhanced migration, recruitment, proliferation and/or differentiation. (B) VEGF actions at the chondro-osseous junction and the metaphysis of growing long bones. The matrix-binding isoforms of VEGF, VEGF164 and VEGF188, are stored in the cartilage matrix after their secretion by hypertrophic chondrocytes. Upon cartilage resorption, mediated by osteoclasts and MMP-9, the released VEGF attracts blood vessels towards the growth plate and stimulates angiogenesis. Indirectly (via the vascular growth) and directly (via VEGFR signaling), VEGF stimulates bone formation by osteoblasts and cartilage resorption and bone remodeling by osteoclasts, thereby coordinating the conversion of cartilage into bone at the chondro-osseous junctions and stimulating the growth of the long bones. Figure adapted from [21,177].

including in cell types present in the bone environment, such as hematopoietic stem cells (HSCs) [67], endothelial cells [68], and osteoblasts [69]. A cell-autonomous role of VEGF in chondrocytes, e.g. contributing to their survival, has also been suggested, although the known signaling receptors VEGFR1 and VEGFR2 are not expressed in cartilage [14,15,70].

Thus, besides inducing angiogenesis in the perichondrium, VEGF appears to play a broader and synchronizing role in the coordinated invasion of the endochondral bone template by directly affecting the endothelial, osteoclastic, and osteolineage cell types involved (Fig. 3A).

2.2.2. *Coordinating actions of VEGF at the chondro-osseous junction and metaphysis of growing long bones*

During longitudinal bone growth following the establishment of the primary ossification center, the last row of hypertrophic chondrocytes of the growth cartilage at the chondro-osseous junction is continuously replaced with trabecular bone that increasingly fills the metaphysis. This progressive turnover of cartilage into bone is driven by the unceasing capillary invasion of the hypertrophic chondrocytes by angiogenic growth of vessels from the growing bone and marrow cavity (Fig. 3B). These vessels likely correspond to the H-type blood vessels recently described to be comprised of endothelial cells that highly express the markers CD31 (PECAM-1) and endomucin, and that are interacting particularly strongly with Runx2+ and Osx+ osteoprogenitors and sensitive to changes in oxygenation and ageing [71–73]. While these H-type endothelial cells particularly make up the vasculature in the metaphysis and the endosteum, the endothelial cells of the sinusoidal vessels in diaphysis express CD31 and endomucin only weakly and are considered to constitute a distinct type of endothelium (type L) [71–73].

Also here, VEGF provides a major engine to the coordinated decay and resorption of the hypertrophic cartilage matrix, vascular expansion and the formation of trabecular bone (Fig. 3B). Inhibition of VEGF action in juvenile mice, through administration of a soluble VEGF receptor chimeric protein (sFlt-1), impaired vascular invasion of the growth plate, and concomitantly, trabecular bone formation and bone growth were reduced and the hypertrophic cartilage zone became enlarged, likely as the result of reduced osteoclast-mediated resorption [74]. Additional mouse genetic studies exposed the specific roles of VEGF and its major splice isoforms [2,14,25,58,64,69]. Altogether, these studies support the model that VEGF, secreted at high levels by hypertrophic chondrocytes, becomes partially sequestered in the cartilage matrix, particularly the longer VEGF isoforms (VEGF164 and VEGF188) that have strong matrix-binding affinity. The shorter VEGF isoforms (VEGF120 and VEGF164) are largely soluble and diffuse from the cartilage, attracting blood vessels towards the chondro-osseous junction and stimulating endothelial cells to form new blood vessels through angiogenesis. This is indirectly associated with increased delivery of osteoclast and osteoblast progenitors. The osteoclasts and osteoclast-derived MMP-9 [75,76] can release more matrix-bound VEGF from the cartilage that is being resorbed, creating a positive-feedback system. Moreover, VEGF also

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has chemo-attractive activity in stimulating osteoclast invasion of cartilage, and enhances osteoclast differentiation, survival, and resorptive activity [76,77]. The osteoblasts on their turn deposit bone on the remnants of the cartilage matrix, in part stimulated directly by VEGF signaling, which enhances the recruitment and differentiation of osteoblasts [21,54]. This model reconciles the data of a large set of mutant mouse models and in vitro studies, which altogether identified VEGF as a crucial physiological driver of the endochondral turnover process that progressively transforms cartilage into bone at the metaphyseal growth plate during skeletal growth.

2.2.3. VEGF-induced neovascularization of epiphyseal cartilage mediates secondary ossification center development

Around postnatal day 5 (P5) in mice, the process of secondary ossification center formation is initiated. At the ends of the long bone, islands of hypertrophic chondrocytes appear within the round chondrocyte layers of the growth cartilage, located close to the articular surfaces (see Fig. 2). It is unclear what triggers the local and patchy differentiation of chondrocytes, but the PTHrP-IHH pathway is likely to be involved (see below). As always, the transition to hypertrophy is associated with the expression of typical hypertrophic chondrocyte markers such as collagen type X and VEGF. The appearance of the hypertrophic cartilage islands is closely followed by the formation of invaginations in the cartilage, the so-called cartilage canals, containing blood vessels and other cells. These tissue-invading structures subsequently expand and fuse to form the secondary center of ossification. As a result, true growth 'plates' comprised of chondrocytes and cartilage matrix are left in between the metaphyseal and epiphyseal bone centers, mediating further postnatal bone lengthening until growth ceases at puberty.

At present, it has not been fully established whether the signals triggering the invasion of the epiphyseal cartilage by blood vessels are coming from the cartilage proper, but it seems likely that chondrocyte-derived VEGF plays a central role also in this phase of cartilage neovascularization and turnover into bone (Fig. 2). In particular, the soluble isoforms VEGF120 and VEGF164 are required for epiphyseal vascularization and induction of secondary ossification in growing long bones. $VEGF^{188/188}$ mice (the knock-in model mentioned before, in which the endogenous VEGF gene is replaced by VEGF188-encoding cDNA, such that the other two major VEGF isoforms VEGF120 and VEGF164 are no longer expressed) showed strongly reduced vascularity around the growth cartilage, impaired invasion of vascular canals into the epiphysis in early postnatal life, and drastically impaired formation of the epiphyseal bone center, associated with a severe knee joint dysplasia and dwarfism [14].

Besides VEGF-mediated vascular invasion of cartilage, collagenolytic actions by membrane type 1-MMP (MT1-MMP, also known as MMP-14) mediating matrix remodeling and angiogenesis have also been implicated in the formation of the secondary ossification center [78,79]. Nevertheless, this aspect of the endochondral ossification process remains far from being understood in all its cellular details and regulatory mechanisms. It will be of interest to see more work in the future characterizing these events and increasing our knowledge of the processes leading to the establishment of the secondary centers of ossification.

3. Chondrocyte-driven regulation of osteoblast differentiation in surrounding tissues

Endochondral ossification during development requires a tightly orchestrated progression of chondrocyte differentiation and osteoblastogenesis in the adjacent perichondrial tissue. A highly coordinated molecular crosstalk between the cell types involved is responsible for the aligned development of cartilage and bone. The details of this interplay are only beginning to be unraveled, but key roles are played by secreted signals such as Indian hedgehog (IHH), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs). In addition, the processes of chondrocyte turnover, osteoblast differentiation, recruitment, and bone-forming activity in the perichondrium and the developing bone center is coupled to the vascularization of the bone and influenced by VEGF signaling as well as by angiocrine signals emanating from the vasculature (also see section 4).

3.1. Perichondrial osteoblastogenesis

The first osteoblasts differentiating in the developing long bones appear in the perichondrium. In fact, early perichondrial cells are thought to be derived from bipotential osteochondroprogenitors, capable of differentiating into either chondrocytes or osteoblasts. A transcriptional program directed by the transcription factors Runx2 and Osx, and the transcriptional co-activator β-catenin (a key mediator of canonical WNT signaling) is essential for the commitment of the mesenchymal precursor cells to the osteoblastic lineage [80] (see Fig. 4). Committed osteoprogenitors or pre-osteoblasts can subsequently differentiate further into mature osteoblasts, increasingly expressing bone matrix proteins including type I collagen (COL1), osteopontin and osteocalcin. The mature osteoblasts deposit and mineralize a sheath of bone matrix called the bone collar, which forms the initiation site of the later cortical bone, around the mid-part of the cartilage model. At least a subset of perichondrial osteoprogenitors, however, does not differentiate to mature osteoblasts in the perichondrium. Instead, these cells remain in the perichondrium as immature osteoprogenitors, until at least a subset of them co-invades the mid-portion of the cartilaginous bone template together with the blood vessels during the initial formation of the primary ossification center, as outlined earlier in this review [55]. These perichondrium-derived osteoprogenitors will populate the nascent primary bone center, where they either remain as rather undifferentiated stromal cells or differentiate to bone-forming osteoblasts [55]. The matured cells deposit mineralized bone matrix on the remnants of calcified cartilage, thereby generating the primitive trabecular bone inside the bone shaft. Thus, the perichondrium is considered a primary source of osteoblasts during endochondral ossification [55,56,81]. In addition to perichondrial osteoprogenitors, recent studies provided evidence that also chondrocytes themselves can contribute to the osteoblast pool in the metaphysis. Indeed, lineage tracing strategies tracking the fate of chondrocytes, by making use of cartilage-specific Cre and CreERt mouse lines driven by the *Aggrecan* (*Agc1*) or *COL10a1* gene promoters, indicated that at least a subset of growth plate chondrocytes can give rise to cells expressing osteoblast markers and populating the trabecular bone region below the chondro-osseous junction, possibly through a process of trans-differentiation [82,83]. Thus, the ultimate fate of the growth plate chondrocyte may not necessarily be to undergo apoptosis at the end of the hypertrophic differentiation program as most generally assumed, but further research will be needed to elucidate the specifics, the magnitude, and the regulation of the alternative fates of chondrocytes during endochondral ossification.

The early osteoblastogenesis processes in the perichondrium have been relatively well characterized, particularly through the generation and investigation of genetically modified mouse models, and the importance of the key transcription factors Runx2 and Osx, and transcriptional co-activator β-catenin, is well established.

3.1.1. Runx2

Runx2 (previously known as core-binding factor a1 (Cbfa1)), a transcription factor of the ancient runt family, is absolutely essen-

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tial for the induction of osteoblast differentiation and endochondral and intramembranous bone formation. Runx2-deficient mice lack osteoblasts and do not form bone at all; instead a completely cartilaginous skeleton develops without any true bone matrix [84,85]. In heterozygous (haploinsufficient) Runx2 mutant mice, the defect in osteoblast differentiation is limited to intramembranous bones [84]. The resulting phenotype in these mice closely resembles the cleidocranial dysplasia (CCD) syndrome in humans, a dominantly inherited developmental disorder of bone, in which *RUNX2* was found to be mutated in most patients [86,87]. Consistent with its function as an early transcriptional regulator of osteoblast differentiation, Runx2 is an early molecular marker of the osteoblast lineage, being highly expressed in perichondrial mesenchyme and in all osteoblasts [88,89]. Hypertrophic chondrocytes also express Runx2, and Runx2 plays important roles in cartilage biology [90,91].

Runx2 is both sufficient and essential for differentiation of mesenchymal cells into osteoblasts, and it inhibits their differentiation into adipocytes and chondrocytes. Runx2 mediates osteoblast differentiation by inducing alkaline phosphatase (ALP) activity, by regulating the expression of a variety of bone matrix protein genes, and by stimulating mineralization in immature mesenchymal cells and osteoblastic cells [88,89]. Furthermore, Runx2 regulates the expression of RANKL and OPG in osteoblasts, thus affecting osteoclast differentiation [92,93].

The DNA-binding sites of Runx2 have been identified in major osteoblast-specific genes, including the genes that encode collagen type 1, osteopontin, osteonectin, bone sialoprotein, osteocalcin, and Runx2 itself, and Runx2 induced the expression of these genes or activated their promoters in vitro [80]. Runx2 transcriptional regulation of different stages of osteoblast differentiation is very complex and involves interactions with a myriad of transcriptional activators, repressors and other co-regulatory proteins. The current model is that Runx2 triggers the expression of major bone matrix protein genes and the acquisition of an osteoblastic phenotype at an early stage of osteoblast differentiation, while inhibiting the late osteoblast maturation stages and the transition into osteocytes. As such, Runx2 may play an important role in maintaining a supply of immature osteoblasts [88,89].

3.1.2. Osx

Runx2 also regulates the expression of Osx (encoded by the *Osx* or *Sp7* gene), an SP family transcription factor with three zinc-finger motifs. Osx is expressed in osteoprogenitors, in osteoblasts, and at a lower level in pre-hypertrophic chondrocytes. Similar to Runx2-deficient mice, mice lacking Osx showed complete lack of osteoblasts and absence of both intramembranous and endochondral bone formation [94]. Thus, Osx represents another transcription factor that is essential for osteoblast differentiation. Because Runx2 is expressed in the mesenchymal cells of Osx-null mice but Osx is not expressed in Runx2-null mice, it can be concluded that Osx acts downstream of Runx2 [94]. Furthermore, the *Osx* gene contains a consensus Runx2-binding site in its promoter region [95]. The transcriptional activity of Osx involves its interaction with NFATc1, cooperatively forming a complex that binds to DNA and induces the expression of the *COL1a1* gene [96]. Osx may be important for directing precursor cells away from the chondrocyte lineage and toward the osteoblast lineage. Expression of genes characteristic of mature osteoblasts (such as those encoding bone sialoprotein, osteopontin, osteonectin, and osteocalcin) was absent in cells surrounding chondrocytes in *Osx*-null mice, and instead these cells express genes characteristic of chondrocytes (*SOX9*, *SOX5*, *COL2a1)* [94]. Osx has also been reported to inhibit chondrogenesis in vitro [97,98]. Overall, it is currently thought that Runx2 has a crucial role in the earliest determination stage of the osteoblast lineage, driving mesenchymal progenitors to become osteoprogenitors, while Osx regulates at a later stage the differentiation of osteoprogenitors to functional, bone-forming osteoblasts expressing high levels of osteoblast markers.

3.1.3. β-catenin

A third crucial osteoblastogenic factor acting at the transcriptional level is β-catenin. β-catenin represents a key mediator of canonical signaling by WNTs, a large family of secreted growth factors (19 different members in mouse and human genomes) that play essential roles in multiple developmental processes, adult tissue maintenance, and cancer [99]. During the past decade, canonical WNT signaling has been shown to play a significant role in the control of osteoblastogenesis and bone formation [100,101].

WNTs can transduce their signals through several different downstream signaling pathways, of which the canonical WNT/β-catenin pathway is the best understood [99,102]. Central to this pathway is the regulation of the protein stability of β-catenin. In the absence of WNTs, cytoplasmic β-catenin is constitutively degraded through its phosphorylation by glycogen synthase kinase 3-β (GSK3-β) in a large protein complex brought together by AXIN and adenomatous polyposis coli (APC) [99]. Phosphorylated β-catenin is recognized by a β-transducin repeat containing protein (β-TrCP) that targets it for proteasome-mediated degradation. In the presence of WNT stimulation, the WNT ligands bind to two synergistically acting families of WNT (co-)receptors: the Frizzled (Fz) receptor family members and low-density lipoprotein receptor-related proteins (LRP5 or LRP6). This interaction results in an inhibition of β-catenin degradation; the β-catenin protein is stabilized, accumulates in the cytoplasm and translocates to the nucleus. Here, it interacts with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA-binding transcription factors to regulate the expression of downstream target genes [99]. Besides its actions as a transcriptional coactivator, β-catenin is also involved in cell–cell adhesion by binding to cadherins.

Genetic studies analyzing conditional β-catenin loss- and gain-of-function mouse models provided compelling evidence that β-catenin is a crucial transcription factor determining the osteoblast lineage commitment of mesenchymal progenitors. During development, β-catenin is indispensible for suppressing chondrocytic differentiation of bipotent osteochondroprogenitor cells, and for stimulating them to differentiate into mature osteoblasts [103]. Inactivation of β-catenin in mesenchymal progenitor cells blocked osteoblast differentiation, and mesenchymal cells in the perichondrium and calvarium differentiated into chondrocytes instead [104] Hu, 2005 #436;Hill, 2005 #6278;Rodda, 2006 #6292}. Combined deletion of the key WNT co-receptors Lrp5 and Lrp6 in the embryonic mesenchyme largely recapitulated the loss of β-catenin, the phenotypes being characterized by impaired osteoblast differentiation in the developing mutant mouse skeletons [105]. In postnatal life, deletion of the gene encoding β-catenin in Osx-expressing osteoprogenitors inhibited their differentiation into mature osteoblasts, and instead induced the expression of adipocytic markers [106,107]. Inactivating β-catenin in more differentiated cells of the osteoblast lineage, mature osteoblasts or osteocytes, resulted in low bone mass phenotypes in mice particularly by increasing osteoclastogenesis and bone resorption; the underlying mechanism involved impaired expression of OPG, an inhibitor of osteoclast formation, in the absence of β-catenin in differentiated osteoblast lineage cells. Thus, β-catenin also plays an important role in the coupling between osteoblast and osteoclast activity during bone remodeling [108,109].

The initiation of this comprehensive osteoblastogenic transcriptional program in the perichondrium is at least in part directed by the adjacent chondrocytes in the growth cartilage. One signal that plays a key role in the crosstalk between chondrocytes in developing long bones and osteoblast precursor cells in the surrounding

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perichondrium is the secreted molecule IHH, which is produced by the pre-hypertrophic chondrocytes and induces Runx2 expression in adjacent cells as outlined below [110,111].

3.2. Actions of IHH in directing osteoblastogenesis during bone development

IHH, a member of the conserved family of hedgehog proteins, is produced by the pre-hypertrophic and early hypertrophic chondrocytes. Hedgehog proteins, upon binding to the receptor Patched (PTC), signal through the seven-pass transmembrane protein Smoothened (SMO) to regulate gene transcription through both derepression and activation of the GLI family of transcription factors [112]. In endochondral bones the PTC receptor (which is also a downstream target of IHH signaling) is present in chondrocytic and perichondrial domains adjacent to the domain of IHH production, encompassing proliferative chondrocytes, perichondrial mesenchymal cells and osteoblasts. In growth plate chondrocytes, IHH regulates the pace of cellular differentiation via a negative feedback signaling pathway with parathyroid hormone-related peptide (PTHrP) acting through the common PTHrP/PTH receptor (PTHR1), thereby controlling the onset of hypertrophic differentiation [110,111]. In addition, IHH directly stimulates chondrocyte proliferation [113–115]. These actions of IHH in chondrocytes proper are discussed in detail elsewhere in this review series on The Biology and Pathology of Cartilage. Here, we will focus on a third key role of IHH in endochondral bone development, in regulating osteoblastogenesis and bone collar formation in the perichondrium.

3.2.1. IHH induces perichondrial osteoblastogenesis and bone collar formation

The first evidence for this role in vivo came from mice completely lacking IHH (IHH-/- or IHH null mice). In addition to alterations in the cartilage, IHH-/- mice displayed a striking absence of osteoblast differentiation in the perichondrium and a total lack of bone collar formation [113]. In situ hybridization to detect Runx2 mRNA revealed that Runx2 expression in hypertrophic chondro-

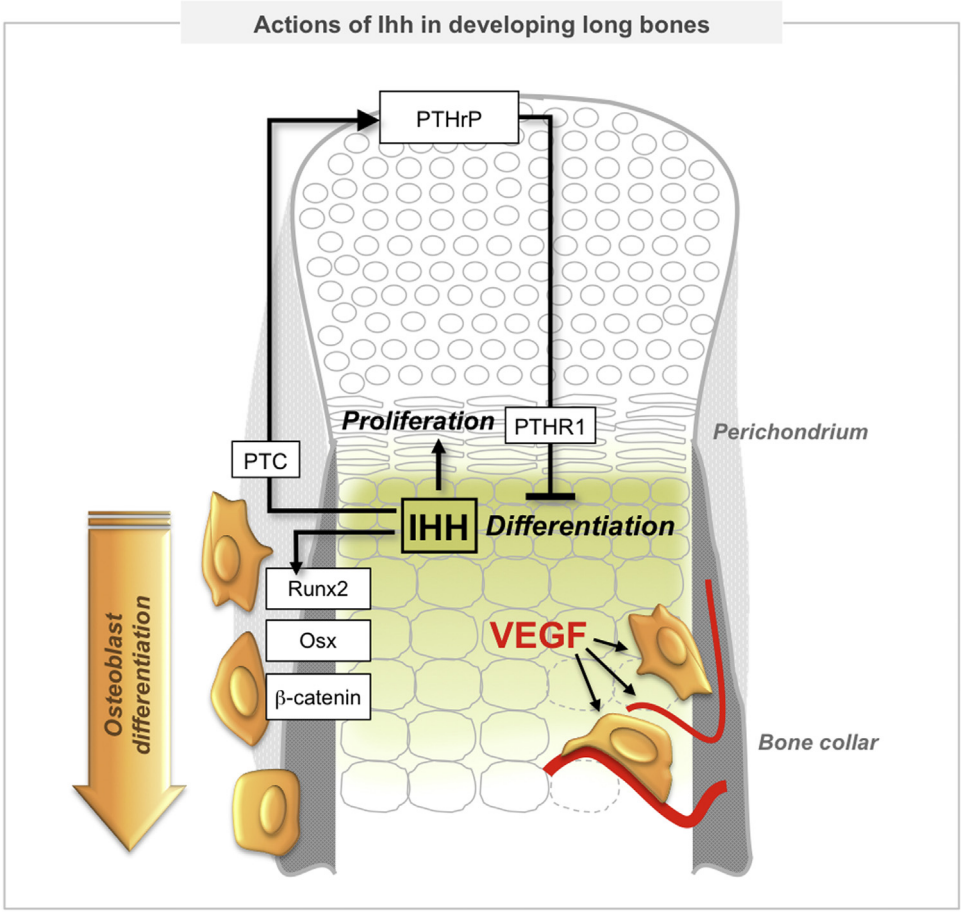

Fig. 4. Actions of IHH in endochondral bone development. IHH, expressed in pre-hypertrophic and hypertrophic chondrocytes (beige colored region), plays key roles in the coordination of chondrocyte proliferation and differentiation, perichondrial osteoblast differentiation and bone collar formation, and vascular invasion of terminally differentiated hypertrophic cartilage. The molecular regulation of the pace of chondrocyte differentiation involves a negative feedback loop with PTHrP, which is most intensely expressed in periarticular chondrocytes close to the end of the bone. Secreted PTHrP acts through its receptor PTHR1, present at low levels in proliferating chondrocytes and intensely in pre-hypertrophic chondrocytes, i.e. the chondrocytes in transit from the proliferative to the post-proliferative state at the interface of columnar and hypertrophic cells. PTHrP-PTHR1 signaling keeps the cells proliferating and delays their hypertrophic differentiation, thereby ensuring an adequate length of proliferative chondrocyte columns and slowing down the generation of cells that can produce IHH. When the distance from the bone end becomes sufficiently large, cells escape the block on hypertrophic differentiation and become IHH-expressing (pre-)hypertrophic chondrocytes and next, VEGF-secreting hypertrophic chondrocytes. IHH signaling is relayed through PTC, present on cells in the regions adjacent to the domain of IHH production, and stimulates the expression of PTHrP. Thus, PTHrP and IHH regulate each other via a negative feedback signaling pathway that controls the cellular differentiation kinetics in the growth plate. IHH thereby indirectly controls also the expression of hypertrophic chondrocyte signals such as VEGF and the initiation of cartilage invasion by blood vessels and perichondrial osteoprogenitors. Independent of PTHrP, IHH also directly stimulates chondrocyte proliferation, and converts mesenchymal progenitor cells in the perichondrium into osteoblasts by inducing the expression of Runx2 in these cells. Perichondrial osteoblast differentiation is transcriptionally driven by Runx2, Osx and β-catenin; by launching this differentiation program in domains adjacent to IHH production, IHH determines the site of bone collar formation. Thus, IHH is a central coordinator of endochondral turnover of cartilage into bone.

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cytes was maintained in IHH null skeletal elements, whereas the signal was undetectable in the surrounding perichondrium. Expression of the mature osteoblast marker osteocalcin was also absent in the IHH-deficient mice, whereas control mice displayed evident expression of Runx2 and osteocalcin in the perichondrial bone collar region [113]. Thus, deficiency in IHH led to an early arrest in osteoblast lineage cell differentiation.

Interestingly, very elegant experiments using chimeras that contained either PTHR1-/- cells or PTHR1-/-;IHH-/- double mutant cells indicated that ectopic bone collar fragments were formed adjacent to ectopic hypertrophic chondrocytes, but only when they expressed IHH [116,117]. In these chimeric embryos, specifically the cells lacking PTHR1, which were dispersed among the wild-type cells throughout the growth plate, escaped the PTHrP-mediated block of differentiation and became hypertrophic ectopically. This was associated with expression of IHH by those cells and with the formation of bone collar in the perichondrial tissues adjacent to the ectopic hypertrophic chondrocytes. In growth plates containing cells that lacked both PTHR1 and IHH, ectopic hypertrophic chondrocytes still formed but the ectopic bone collar formation was absent, indicating that this feature was dependent on IHH [116,117].

A subsequent study analyzed whether IHH signaling is directly required for osteoblast differentiation, by targeting the signal transducer SMO in genetically modified mice [118]. By using different Col2-Cre lines, with varying degrees of efficiency and specificity for targeting perichondrial cells and/or chondrocytes, it was shown that conditional inactivation of SMO from perichondrial cells prevented the formation of a normal bone collar. Furthermore, analysis of chimeric embryos composed of wild-type and SMO-/- cells revealed that SMO-/- perichondrial cells failed to contribute to osteoblasts in either the bone collar region or the primary spongiosa, but generated ectopic chondrocytes instead [118].

Altogether, these experiments convincingly showed that IHH directs the site of osteoblast differentiation by localized signaling from its pre-hypertrophic/hypertrophic chondrocyte site of production to the neighboring perichondrium, where IHH-induced direct actions provide control of osteoblastogenesis and bone collar formation (Fig. 4) [112–114,116,118–120]. Of note, osteoblast differentiation does not seem to require IHH in all settings to the same extent. For instance, while IHH−/− mice showed complete lack of bone in the endochondral skeleton, intramembranous ossification in the bones of the skull appeared much less affected by the absence of IHH [113], although IHH does serve as a positive regulator of osteoblast differentiation in intramembranous bones too [121]. Also, in a conditional knockout model (Prx1-Cre-driven inactivation of IHH in the limbs) that allowed survival of the mice to 3 weeks of age, it was found that although bone formation was initially delayed, osteoblast differentiation and bone formation could occur in IHH deficient bones postnatally [122].

3.2.2. Mechanism of action of IHH in the perichondrium and downstream signaling

As mentioned, perichondrial cells are thought to be bipotential osteo-chondroprogenitors; Runx2, Osx, and canonical, β-catenin-mediated WNT signaling are essential for the commitment of the mesenchymal precursor cells to the osteoblastic lineage [80]. Evidence has shown that the early perichondrial progenitors are driven into the osteoblast lineage under the influence of IHH stimulating the expression of Runx2 [113,118,123]. In the absence of IHH, cells of the osteoblast lineage fail to activate the expression of Runx2. Yet, forced expression of Runx2 in the skeletal cells (using a Col2-Cre mouse strain that targets both chondrocyte and osteoblast lineages in the endochondral skeleton to activate a conditional Runx2 transgene engineered into the Rosa26 locus) restored bone formation in $Runx2^{-/-}$ embryos, but not in $IHH^{-/-}$ embryos [123]. Thus, the mechanism through which IHH induces osteoblast differentiation appears to require other, yet unidentified, effectors in addition to Runx2. Both the WNT/β-catenin signaling pathway and the BMP pathway have been reported to act downstream of IHH in regulating osteoblast differentiation from perichondrial cells during endochondral ossification [124,125].

The transcriptional upregulation of Runx2 by chondrocyte-derived IHH seems to be mediated particularly by GLI2, one of the GLI family proteins that constitute the downstream transcriptional regulators transducing IHH signals [126]. IHH, like the other hedgehog proteins encoded by the mammalian genome (namely Sonic hedgehog (Shh) and desert hedgehog (Dhh)), signals through a receptor complex consisting of the hedgehog cell surface receptor PTC and SMO, a putative G-protein-coupled seven trans-membrane domain protein. In the absence of ligand, PTC represses the activity of SMO; when a hedgehog protein binds to PTC, the inhibition of SMO by PTC is released. SMO undergoes a conformational change, activating signaling activity through both derepression and activation of GLI family proteins. The GLI family transcriptional regulators, consisting of three related transcription factors termed GLI1, GLI2, and GLI3 in mammals, represent the key intracellular transducers of IHH signaling by translocating to the nucleus to control the transcription of the IHH target genes [127]. GLI3 and to a lesser extent GLI2 can be proteolytically processed into short repressor proteins [128,129].

Skeletal abnormalities have been described in mutant mice lacking the GLI proteins [112]. GLI3 seems to mainly act as a strong repressor of IHH signals in chondrocytes. Loss of GLI3 causes severe polydactyly in mice [130], but in IHH-/- mutants it restored the chondrocyte proliferation defect and delays the accelerated onset of hypertrophic differentiation [131,132]. Furthermore, the expression of the IHH target genes PTC and PTHrP was reactivated in IHH−/−;GLI3−/− mutants [132]. Thus, de-repression of the GLI3 repressor is thought to be the predominant downstream mechanism by which IHH controls chondrocyte proliferation and maturation.

In contrast, GLI2 seems to act predominantly as an activator by inducing the expression of IHH target genes in skeletal tissues [133]. GLI2 mutant mice exhibit skeletal abnormalities including short limbs with delayed endochondral ossification [134,135]. Upon IHH signaling, processing of GLI2 is inhibited and the full-length protein functions as an activator of transcription. GLI2 mediates IHH-induced osteoblast differentiation in mesenchymal cell lines by associating with Runx2 and stimulating its expression and function [126], as well as by inducing BMP2 expression [136]. Moreover, GLI2 activator alone was found to be sufficient in vivo to induce vascularization of the hypertrophic cartilage in IHH null mice, but required simultaneous removal of GLI3 to restore osteoblast differentiation [133,137].

GLI1 seems largely functionally redundant with its close relatives, as GLI1-/- mice do not show any gross abnormalities in adulthood. Yet, bone formation was impaired in GLI1-/- fetuses compared with wild-type fetuses and GLI1 seems to participate in the osteogenic response of perichondrial cells to IHH [138,139]. Other downstream regulators of hedgehog signaling that could affect GLI actions are suppressor of fused (Sufu) and Kif7, both of which are also expressed by growth plate chondrocytes [140,141].

3.2.3. Regulation of IHH expression

In the growth plate chondrocytes, the expression of IHH itself is regulated by Runx2 [142–144], which is also expressed in chondrocytes in an IHH-independent fashion [113]. Constitutive expression of Runx2 in non-hypertrophic chondrocytes induced hypertrophic differentiation, IHH expression and endochondral bone formation [143]. Another transcription factor that was found to regulate IHH expression in chondrocytes is ATF4. In vitro assays indicated that

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ATF4 can bind to the IHH promoter and act as a transcriptional activator of IHH in chondrocytes [145]. Ablation of ATF4 in mice led to severe skeletal defects, including a short stature and short limbs with accelerated hypertrophy of chondrocytes and delayed ossification; these abnormalities were associated with reduced IHH expression and signaling [145,146]. When ATF4 was overexpressed selectively in chondrocytes of ATF4-/- mice, the reduced stature, the growth plate defects, and the decrease in IHH expression were all corrected. Surprisingly, chondrocyte-derived ATF4 also restored the osteoblastic phenotype and osteogenesis defects of ATF4-/- mice, which appeared to be mediated through IHH [146]. In recent work the transcription factor core binding factor beta (Cbfβ) was also found to regulate IHH expression [147].

Several secreted signaling molecules regulate the production of IHH, including PTHrP and members of the BMP and FGF families (see below). VEGF has also been suggested to affect the expression of IHH [58]. Additional influences on IHH signaling may be provided by the matrix; for instance, IHH may bind to aggrecan, via chondroitin sulphate side chains, and sulphation of these side chains appears to be required for normal IHH signaling in the murine growth plate [148]. Lastly, for correct IHH signaling also an intact primary cilium has to be present [149].

3.3. Coupling of chondrogenesis, osteogenesis and angiogenesis in endochondral bones

From all the above, it is evident that IHH is a master regulator of endochondral bone development, coordinating the temporal-spatial coupling of chondrocyte proliferation, chondrocyte maturation and osteoblast differentiation in the immediate adjacent perichondrium [127]. Furthermore, IHH functions to synchronize these processes to the vascularization of the endochondral bones, a prerequisite for subsequent bone formation as outlined earlier in this review [21]. Indeed, IHH null mice also displayed a block of vascular invasion of the developing bone template and primary ossification center formation did not occur by the time of their death just after birth [113,150]. This phenomenon cannot be explained by potential consequences of IHH deficiency in the endothelial cells proper, as the same phenotype was observed in mice with conditional inactivation of IHH in Col2-Cre-expressing cells only [151]. Thus, chondrocyte-derived IHH appears to be essential in mediating the invasion of the cartilaginous bone model by blood vessels, which itself is an absolute requirement for endochondral bone formation and growth to proceed.

Two aspects appear absolutely essential for the initiation of cartilage neovascularization in early skeletogenesis, both of which are under the control of IHH (Fig. 4). First, as outlined earlier in this review, cartilage neovascularization is driven by and dependent on high levels of VEGF production by hypertrophic chondrocytes [21]. Since IHH determines the timing of terminal differentiation of chondrocytes to become hypertrophic chondrocytes, it indirectly determines the expression of VEGF. However, although chondrocyte differentiation was delayed at early stages in the IHH mutant mouse models, at later stages hypertrophic chondrocytes were present and found to express markers of terminal differentiation, including matrix metalloproteinase (MMP)-13, osteopontin, as well as VEGF [150,151]. Thus, IHH-/- cartilage can produce the angiogenic signals that are necessary for neovascularization, indicating that additional reasons likely underlie the drastically impaired vascular invasion of IHH-deficient cartilage. This second requirement for rendering cartilage permissive for vascular invasion could be perichondrial osteoblastogenesis and bone collar deposition. Osteoblast differentiation and bone collar formation were lacking in IHH-/- mice; concomitantly, the cartilaginous rudiment remained non-invaded [113,150]. Conversely, in chimera experiments in which IHH was genetically misexpressed in cartilage and ectopic bone collar fragments formed near sites of ectopic IHH production (see before), this was associated with ectopic vascular invasion at multiple sites along the immature cartilage [112,116]. The need for perichondrial osteogenesis prior to vascular invasion is also supported by other studies. For instance, ex vivo cultured bone explants from which the perichondrium had been removed, were no longer able to develop a primary ossification center [56]. Runx2-/- mice, Osx-/- mice, and β-catenin conditional knockout mice, all show a block in perichondrial osteoblastogenesis [84,85,104,152,153], and concomitantly display a near-complete absence of vascular invasion and primary ossification. It remains however difficult to discern the specific role of correct bone collar establishment, given for instance that these osteoblastogenic factors function in chondrocytes as well and transcriptionally regulate VEGF [62,154].

Thus, while it is not fully clear as yet what constitutes the initial trigger that launches the neovascularization of developing bones, genetic studies in mice have begun to shed light on the intertwined molecular regulation of chondrocyte differentiation, perichondrial osteoblastogenesis, and vascular invasion of the cartilage anlagen, with central roles in the crosstalk between the cartilage and the adjacent tissues being played by the secreted signals IHH and VEGF.

4. Osteocrine and angiocrine signaling regulating chondrocyte differentiation and turnover

Besides signals produced by chondrocytes affecting angiogenesis and osteogenesis in the surrounding perichondrial tissues as illustrated above, it is most conceivable that the tight coordination of the developmental cascade in endochondral bones is reliant on a reverse signaling towards chondrocytes as well (Fig. 1). Here, we will briefly touch on some of the signals produced by mesenchymal progenitors and/or osteoblast lineage cells in the perichondrium (osteocrine signals) and by endothelial cells (angiocrine signals), which may influence chondrocyte differentiation and turnover/fate. Our knowledge on this bidirectional crosstalk is, however, still fragmentary and incomplete.

4.1. Crosstalk between the osteogenic perichondrium and the growth plate

Reciprocal signaling by perichondrial cells to control the cartilage mold is thought to occur via the secretion of signaling molecules that regulate chondrocyte proliferation and differentiation, such as PTHrP, WNTs, BMPs, and FGFs (particularly FGF18) [111]. As the PTHrP/IHH negative feedback loop is central to the regulation of chondrocyte proliferation and the pace of chondrocyte differentiation, it is not surprising that several of these secreted molecules may affect the maturation of growth plate chondrocytes by influencing the production of IHH and interacting with the PTHrP/IHH pathway [155–157]. In fact, each of these pathways has multiple mechanisms for interacting with each other and coordinately regulate chondrocyte proliferation, IHH production, and terminal hypertrophic differentiation. As indicated in Fig. 5, perichondrial cells, chondrocytes, and osteoblasts express multiple BMPs, BMP receptors, and the BMP antagonists noggin and chordin, as well as FGF18 and FGF receptors [158]. Generally, the FGF and BMP pathways appear to antagonize each other during chondrocyte differentiation [159] (Fig. 5).

4.1.1. BMPs

BMPs, secreted proteins belonging to the TGFβ superfamily, transduce signals through their serine/threonine kinase receptors (homomeric or heteromeric complexes composed of type I and type II BMP receptor subtypes), which leads to the activation of

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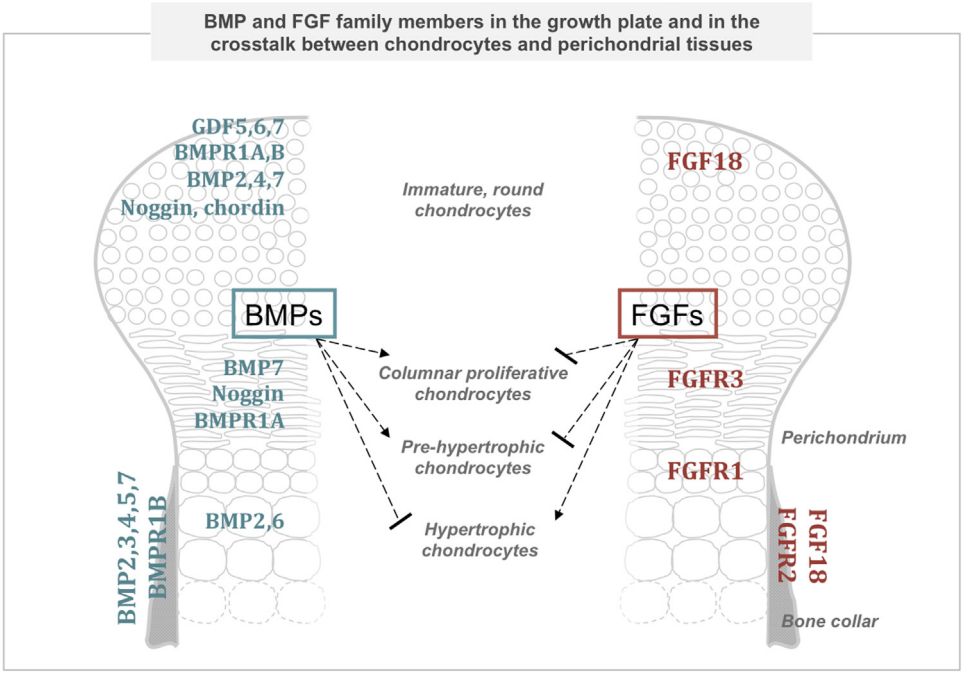

Fig. 5. BMP and FGF family members in the crosstalk between cartilage and surrounding perichondrial tissues. Several BMP and FGF family members (ligands, receptors, antagonists) are expressed in specific domains of the growth cartilage (depicted from the periarticular end of the epiphysis (top) towards the metaphysis (bottom) as zones of immature round chondrocytes, strongly proliferating columnar chondrocytes, pre-hypertrophic and hypertrophic chondrocytes) and in the surrounding perichondrial tissues. The precise functions of all individual family members are not completely understood at present, but overall the BMPs and FGFs are thought to modulate chondrocyte proliferation, differentiation to pre-hypertrophic chondrocytes (and their expression of IHH), and terminal hypertrophic differentiation, in ways that are largely antagonistic as indicated in the scheme. Figure modified from [158].

intracellular proteins of the Smad family that relay the BMP signal to target genes in the nucleus [88,160]. Around 20 BMP family members have been identified to date. Several of the various BMP ligands, BMP receptors, and BMP antagonists are expressed in specific layers of the growth plate, in the perichondrium, and in osteoblasts [158]. Studies using genetically altered mouse models showed that BMP signaling through type I receptors in growth plate chondrocytes stimulates chondrocyte proliferation and survival, while concomitantly delaying the conversion to terminal hypertrophic differentiation [159] (Fig. 5), findings that confirmed previous results obtained in limb cultures [155–157]. Of note, in developing bones BMPs are particularly important in the very early stages of limb patterning and skeletal morphogenesis, when they play roles in determining the size and shape of the mesenchymal condensations and in converting the condensing mesenchyme into chondrocytes [161–163]. But BMPs also function in the subsequent processes of chondrogenesis and osteogenesis and later in life during repair of bone defects [164]. The precise roles of BMPs in these processes have, however, been difficult to establish, given that genetic modification of BMP signaling is often associated with severe early defects and lethality (as for instance seen with loss of BMP2 or BMP4), potential functional redundancy among family members (around 20 BMP family members have already been identified), and difficulties to discern whether effects on bone formation may be direct versus consequential to alterations in chondrogenesis [164,165]. Ongoing studies employing conditional (site-specific and/or inducible) mutagenesis strategies will undoubtedly be very informative.

4.1.2. FGFs

The FGF pathway similarly involves multiple ligands and receptors that take part in regulating skeletal development, with most notable functions in regulating chondrogenesis assigned to the ligands FGF18 and FGF9 and the receptor FGFR3 that they activate [166–169]. Activating mutations in the human *FGFR3* gene cause dominantly inherited dwarfing chondrodystrophies, which is due to impaired chondrocyte proliferation [170]. FGFR3 is expressed on proliferating chondrocytes and provides a master block on chondrocyte proliferation, through FGFR3-mediated activation of STAT1 activating the cell cycle inhibitor $p21^{Waf1/Cip1}$ [171]. Accordingly, FGFR3 inactivation in mice increased chondrocyte proliferation and prolonged growth [172,173]. These findings were surprising given that FGFs are classically viewed as potent mitogens. The effects of FGF signaling on the differentiation of chondrocytes are less clear. In vitro, FGF signaling accelerated the late steps of chondrocyte hypertrophy [155], but mutant mouse models strongly suggested that FGFR3 signaling in vivo inhibits hypertrophic differentiation of chondrocytes [170,174,175]. Part of the complexity may arise from the fact that the effects of FGFR3 are regulated only in part by direct signaling in chondrocytes, and in part indirectly by FGFs modulating the expression of the IHH/PTHrP/BMP signaling pathways. For instance, mice harboring an activating mutation in *FGFR3* have decreased expression of IHH, PTC, and BMP4, whereas the expression of these genes is upregulated in mice lacking FGFR3 [174,176].

4.2. Angiocrine signaling by skeletal endothelial cells

Invasion of cartilage by blood vessels is inherently associated with turnover of cartilage into bone. Already several decades ago, it has been suggested that the invading endothelial cells may elicit signals that induce apoptosis of chondrocytes; these have, however, remained unidentified to date. Currently, the debate is still ongoing to what extent terminal hypertrophic chondrocytes are destined to undergo apoptosis versus transdifferentiating to osteogenic cells. In either case, it is highly conceivable that hypertrophic chondrocytes are exposed to angiocrine signals, expressed and/or released by the angiogenic endothelial cells at the initial invasion front in the fetal

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bone template and at the chondro-osseous junction of the later growth plate. In addition to the influences of oxygenation, nutrient availability, endocrine factors and osteoblast and osteoclast precursors brought to the invasion region by the vasculature, such angiocrine signals, albeit as yet unidentified, may possibly affect the fate of the chondrocytes and contribute to the turnover of cartilage into bone.

In support of the role of angiocrine signaling in the skeleton, recent work implicated Notch-signaling in endothelial cells in the regulation of endothelial production of Noggin, a major modulator of the BMP pathway, thereby contributing to the control of angiogenic-osteogenic coupling [72]. This study employed mouse models with endothelium-specific and inducible inactivation of target genes by using Cdh5(PAC)-CreERt2 mice [72]. An intense molecular cross-talk between endothelial and osteogenic cells likely determines the close association between the processes of bone formation and vascularization of the ossified tissue (see Fig. 1). Besides noggin, several other locally produced angiocrine signals, secreted by vascular endothelial cells in the bone microenvironment, may influence osteoblast lineage cells and bone formation.

Thus, it may be anticipated that the currently underappreciated role of angiocrine signaling in the skeleton will be resolved in the coming years through the use of creative combinations of site-specific and temporally regulated mutagenesis approaches in mice. This will help to further clarify the molecular underpinnings of the coupling between cartilage neovascularization and cartilage turnover, as well as between angiogenesis and osteogenesis, in bone development, homeostasis and fracture healing.

5. Summary

A large number of studies performed over the past 20 years have established central roles of interacting signaling pathways and molecules in the control of chondrocyte development and the spatiotemporal alignment of hypertrophic chondrocyte maturation with perichondrial osteoblastogenesis and cartilage neovascularization. Chondrocytes direct osteogenesis and angiogenesis in the surrounding tissues to a great extent through their production of IHH and VEGF, and we have come a long way already in elucidating the regulation of their expression, the downstream mechanisms that mediate their effects, and the interactions with other signaling networks. It does remain challenging to dissect the causative and consequential changes associated with genetic mutations in mice when studying the complex process of endochondral bone development, particularly because a variety of cell types are involved, which can be affected both in cell-autonomous and secondary ways, and because several interconnected processes take place in a small window of time within the area of the developing bone. Further studies will help to shed full light on these intertwined processes and their molecular control, helped by advanced strategies for target population-selective and temporally controlled mutagenesis in mice. Expanding this toolbox further will be particularly important for the future unraveling of the reciprocal signaling events, meaning the communication factors that are elicited by perichondrial mesenchymal progenitors and osteogenic cells (osteocrine signals) and by endothelial cells (angiocrine signals), and that likely function in the synchronization of cartilage and bone development. As described, our knowledge on the molecular constituents of this bidirectional crosstalk is likely still fragmentary and incomplete, with much remaining to be learned about the interplay between skeletal progenitors, chondrocytes, endothelial cells, osteoblasts, osteoclasts, and other cell types present in the bone environment.

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